


INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

**PRIORITY DOCUMENT**

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

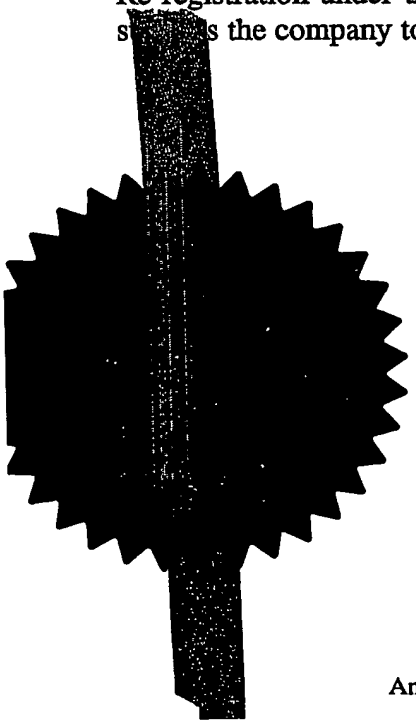


I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely s[illegible]s the company to certain additional company law rules.



Signed Andrew Gerry

Dated 25 November 2003

Patents Form 1/77

Patents Act 1977
(Rule 16)

THE PATENT OFFICE
A
- 6 NOV 2002
LONDON

PATENTS • DESIGNS • COPYRIGHT • TRADE MARKS
The Patent Office

1/77

07NOV02 E761490-1 D00192
P01/7700 0.00-0225885.3

# Request for grant of a patent

*(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)*

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference — N86941

2. Patent application number
*(The Patent Office will fill in this part)* — 0225885.3 — 6 NOV 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

ISIS INNOVATION LIMITED
EWERT HOUSE
EWERT PLACE
SUMMERTOWN
OXFORD, OX2 7SG

Patents ADP number *(if you know it)* 39985640003

If the applicant is a corporate body, give the country/state of its incorporation — UK

4. Title of the invention — QUANTIFYING EXPOSURE TO STRESS

5. Name of your agent *(if you have one)*

J.A. KEMP & CO.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

14 South Square
Gray's Inn
London
WC1R 5JJ

Patents ADP number *(if you know it)* 26001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

| Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
|---|---|---|

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

| Number of earlier application | Date of filing *(day / month / year)* |
|---|---|

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

*a) any applicant named in part 3 is not an inventor, or*
*b) there is an inventor who is not named as an applicant, or*
*c) any named applicant is a corporate body.*
*See note (d))*

YES

**Patents Form 1/77**

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

| | |
|---|---|
| Continuation sheets of this form | |
| Description | 19 |
| Claim(s) | 3 CF |
| Abstract | 1 |
| Drawing(s) | 2 → 2 |

10. If you are also filing any of the following, state how many against each item.

| | |
|---|---|
| Priority documents | |
| Translations of priority documents | |
| Statement of inventorship and right to grant of a patent *(Patents Form 7/77)* | |
| Request for preliminary examination and search *(Patents Form 9/77)* | 1 ✓ |
| Request for substantive examination *(Patents Form 10/77)* | |
| Any other documents *(please specify)* | |

11. I/We request the grant of a patent on the basis of this application.

Signature J.A. Kemp & Co Date 6 NOVEMBER 2002

J.A. KEMP & CO.

12. Name and daytime telephone number of person to contact in the United Kingdom

IRVINE, Jonquil Claire
020 7405 3292

**Warning**

*After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.*

**Notes**

*a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.*

*b) Write your answers in capital letters using black ink or you may type them.*

*c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

*d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

*e) Once you have filled in the form you must remember to sign and date it.*

*f) For details of the fee and ways to pay please contact the Patent Office.*

# QUANTIFYING EXPOSURE TO STRESS

The present invention relates to *in vitro* methods of quantifying exposure to psychological stress in an individual, which may be a human or non-human animal. More particularly, the present invention provides a method of quantifying exposure to psychological stress which relies on measuring the retained ability of neutrophils, preferably neutrophils in a whole blood sample, to exhibit challenge-induced superoxide anion production, i.e. produce a "respiratory burst" in response to *in vitro* activation. Such methodology may also be applied to screen for stress-relieving drugs.

## Background to the invention

Objective, quantitative and practicable measures of psychological stress are pivotal to studies in many branches of vertebrate biology, including wildlife conservation and management, and are also relevant to the farming industry, animal welfare and veterinary services. Techniques for quantifying levels of human stress are important from a medical perspective, but are also of interest for occupational safety and health. An estimated 1 in 10 people in the UK suffer from work related stress (Health & Safety Executive Information sheet: 1/01/EMSU updated February 2002) at a cost of £3.7 billion to society. Stress-related conditions or effects include immune disorders, cardiovascular disease, muscoskeletal and psychological disorders, workplace injury and ulcers.

Various methods have previously been applied in an attempt to assess stress responses, including the perceived stress questionnaire (Cohen et al., J. Health & Social Behaviour (1983) 24, 385-396), measurement of cortisol and hormonal levels in blood, urine, saliva and faeces (Beerda et al., Horm. Behav. (1996) 30, 272-279), haematological values (e.g. Millspaugh et al., Can. Field-Nat. (2000) 114, 196-200) and behavioural observations (reviewed by Rushen in The Biology of Animal Stress Basic Principles And Implications For Animal Welfare (2000) ed. Moberg & Mench, Wallingford:CABI Publishing). However, these approaches have various drawbacks,

and may be inappropriate to use in certain situations. The perceived stress questionnaire is subjective, and so cannot provide an objective measure of stress. Furthermore, questions may not always be answered honestly, for example due to cultural reasons, and this measure of stress cannot be used for testing animals or birds. Measurements of cortisol and hormonal levels in, for example, blood and urine, are often used to assess stress in animals, but these measures are not ideal since their levels change throughout the year. There are no absolute terms of reference, and this type of testing is time consuming. Behavioural observations are also often subjective and difficult to quantify.

More recently, it was shown that exposure of human volunteers to a short term mental stressor leads to increased superoxide anion production being observable in neutrophils present in peripheral blood samples (Ellard et al., Inter. J. Psychophys. (2001) 41, 93-100). It has now been found that exposure of both animals and humans to psychological stress can be rapidly and readily quantified by relying on measurement of the retained capacity of neutrophils in peripheral blood samples to produce superoxide anions in response to a challenge by phorbol myristate acetate (PMA), a known chemical-inducer for activating neutrophils (Hu et al., Cell Signal (1999) 11, 355-360). By this means, for the first time, it has proved possible to obtain a quantitative measurement of copying capacity of animals and humans for known or suspected psychological stressors.

**Summary of the invention**

The method of the invention for quantifying stress is based on direct measures of a cellular response which forms part of the armoury of the immune system. It relies on the ability of individuals to mount a challenge-induced immune response after a potentially stressful event. Each individual's capacity to respond to immune challenge is compared with its own baseline level of immune system activity. After exposure to a known or suspected psychological stressor, superoxide anion production in neutrophils is stimulated *in vitro*, and the capacity of neutrophils to produce further superoxide is measured, in effect determining the extent to which

superoxide production has been diminished by the known or suspected stressful event. The ability of neutrophils to respond to such *in vitro* challenge after a stressful event is defined as the individual's coping capacity. Individuals with a higher coping capacity have a greater potential superoxide production and, physiologically, are better able to cope with bacterial challenge after stress. Therefore coping capacity is an *in vitro* assessment of the individual's current physiological status.

In its broadest aspect, the present invention therefore provides a method for determining whether an individual, which may be a mammal, including a human, or a bird, is experiencing changed physiological status arising from a psychological stressor, the method comprising:

(a) contacting a test sample comprising neutrophils obtained from said individual with an inducer capable of stimulating superoxide production in neutrophils under conditions suitable for such stimulation;

(b) determining the increase in superoxide production above basal in said test sample after a time period when neutrophils of the same species in a control sample, which are free or substantially free of stress-induced activation or at least derived from one or more individuals exposed to the same regime minus a factor to be tested as a psychological stressor, will exhibit superoxide production under the same *in vitro* conditions; and

(c) comparing the increase in superoxide production above basal observed in said test sample with the increase in superoxide production above basal observed in a control sample as defined in (b) above under the same conditions;

wherein lower superoxide production in said test sample is indicative of the effect of a psychological stressor on the individual's physiological status. As indicated above, residual capacity of neutrophils above basal for *in vitro*-induced superoxide induction can be termed "coping capacity".

The control neutrophils may not be entirely free of stress-induced activation but will necessarily be obtained from the same individual or an individual of the same species prior to exposure of the individual to a suspected or known psychological stressor selected for study. For example, Example 1 illustrates application of such methodology to badgers where the additional stress-inducing effect of transport was quantified compared to mere badger capture based on comparing the residual capacity for challenge-induced activation of neutrophils from transported badgers with such residual capacity of neutrophils from captured but not transported badgers (the controls).

The samples employed in a method of the invention may be simply whole peripheral blood samples and hence such a method provides rapid results and can be utilised in the field or laboratory. Such a method can be used to elucidate psychological stressors or to determine whether an individual is suffering from recent or continued exposure to a psychological stressor. As indicated above, most importantly, for the first time the present invention provides a means of quantifying the ability of individuals to cope with known or suspected psychological stressors.

Hence, in a preferred embodiment, there is provided a method for determining the coping capacity of an individual for exposure to a psychological stressor, wherein prior to step (a) in a method as defined above said individual is exposed to said psychological stressor for a time period whereby neutrophils in an individual of the same species who is susceptible to stress induced by said stressor will exhibit increased superoxide production and wherein the degree of further *in vitro* induced superoxide production in said test sample above basal determined in step (c) is a measure of coping capacity.

**Brief description of the drawings**

Example 1 illustrates use of the methodology of the invention to test the prediction that badgers (*Meles meles)* have a lower coping capacity when they are subjected to

trapping and then transport than when they are trapped but do not experience the additional stress of transport with reference to the following figures:

Figure 1 shows coping capacity (solid lines, mean ± SE) in 8 badgers after transport (square), and 8 badgers without transport (circle) as determined by chemiluminescence measurement of superoxide production in whole blood after challenge with PMA. Dashed lines represent basal leucocyte activity.

Figure 2 shows coping capacity per leucocyte, calculated as relative light units divided by the number of leucocytes ($10^9$/L). Solid lines represent coping capacity (mean ± SE) in badgers after transport (square), and without transport (circle). Dashed lines represent basal per-leucocyte activity.

**Detailed description of the invention**

A method of the invention may be a carried out on neutrophils obtained from an individual in any manner whereby the neutrophils are in a sample suitable for chemical-induced superoxide production but will most preferably for convenience be applied to neutrophils in a whole blood sample. For example, in the case of a human, such a sample may be obtained by a simple finger prick. If need be, blood samples for use in a method of the invention may be treated with an anticoagulant. While a whole blood sample may be utilised directly, with or without an anticoagulant, it will be appreciated that a blood fraction comprising neutrophils may alternatively be employed if desired. For example, a sample comprising isolated leucocytes may be employed.

A sample to be tested, directly or after further processing, may be obtained during or shortly after exposure of the individual of concern to a suspected or known psychological stressor. Where one or more samples is taken after exposure to a known or suspected psychological stressor, the initial such sample will generally be obtained as soon as possible. Multiple samples from the same individual may be tested taken at different time points during and/or after exposure to a suspected or known psychological stressor, e.g. within 1 to 5 minutes, 10, 15, 20 or 30 minutes of

exposure to the suspected or known psychological stressor. Multiple samples may be tested which have been obtained at different time points after exposure to a psychological stressor in order to determine peaking of the stress response and /or the individual's recovery rate. Alternatively, for simplicity and speed, an individual's coping capacity for a known stress factor may be tested at a single time point after exposure to the stress factor. When testing for stress in animals, the sample may be obtained whilst an animal is under anaesthesia.

A sample to be tested in accordance with the invention may be contacted with any chemical inducer which is capable of stimulating superoxide production in neutrophils. The inducer used in the method may be preferably phorbol myristate acetate (PMA), more particularly, for example, the microbial product phorbol 12-myristate 13-acetate obtainable form Sigma. However, alternative inducers which might be employed are well-known. They include N-Formyl-Met-Leu-Phe (FLMP chemotactic peptide), zymosan, lipopolysaccharide or adrenaline. Suitable lipopolysaccharide is obtainable from the cell wall of a gram negative bacterium, for example *E. coli*. A suitable concentration of inducer and period and temperature of induction may determined if need be by simple experimentation. In the case of PMA, typically it may be added to a whole blood sample at about $10^{-6}$ M to $10^{-3}$ M and the temperature of the sample maintained at about 37°C. Superoxide production may be measured at various time points or a suitable single measurement time point may be determined.

The production of superoxide in response to the inducer may be measured by any known means. Preferably, however, chemiluminescent measurement will be employed for convenience coupled with high sensitivity. For this purpose, a suitable amplifier such as luminol (5-amino-2,3-dihydrophthalzine) or isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) may be utilised to detect superoxide as described by Hu et al., Cell Signal (1999) 11, 355-360. Chemiluminesence may be conveniently measured using a portable chemiluminometer. In this way, coping capacity for stress factors may be readily quantified even outside of a laboratory.

As indicated above, the level of superoxide production may be measured at one time point after the addition of inducer to the sample, or at multiple time points. Typically, superoxide production is measured at multiple time points at intervals of between 1 and 10 minutes, for example every 5 minutes, after addition of inducer, over a total time period of between 5 minutes and 1 hour, for example over 30 minutes. Preferably, the level of superoxide production is measured after a time period when control neutrophils of the same species, which are free or substantially free of stress-induced activation, will exhibit maximal superoxide production under the same *in vitro* conditions. This time point can be determined by testing samples obtained from individuals who have not been exposed to a psychological stressor, and measuring superoxide production at various time points after addition of inducer. At the preferred time point, it would be expected that the difference between superoxide production in a sample from an individual exposed to stress, and one who has not been exposed to stress, would be at its greatest. Therefore the test is ideally more sensitive to any changes as a result of stress.

The production of superoxide in response to the inducer is compared to the basal superoxide production in the absence of inducer of the same sample, or a further sample taken from the same individual. Basal measurements enable such factors as individual differences in the production of reactive oxygen species, contribution of differences in haematocrit and haemoglobin, and differences in the number of circulating leukocytes to be taken into account. By comparing these two levels, it is possible to determine the amount of superoxide produced above the background or basal level.

A method of the invention may be used to assess individual responses to the same psychological stressor and compare coping capacities between individuals for a particular psychological stressor. For example, two or more individuals, e.g. two or more humans, may be exposed to a known stressor, and their coping capacity measured at the same time point in accordance with the preferred embodiment of the invention set out above. The results can then be compared to find which of the tested individuals has coped the best with exposure to the psychological stressor. An

individual with a lower coping capacity as determined by *in vitro* induced superoxide stimulation in neutrophils is less able to cope with the stressor than an individual with a higher coping capacity determined in the same manner after exposure to the same stressor. This method would therefore be of particular use in situations where, after a stressful event, a rapid assessment of the individual's ability to cope is required or where it is desired to select individuals on basis of better ability to cope than others with a recognised psychological stressor. Such a method could, for example, form part of a job selection procedure for human individuals where the job is known to entail exposure to psychological stress factors.

A method of the invention may also be used to compare the effects of different stressors on the same individual or group of individuals, for example a sample of individuals from the same species. In this case, the individual's coping capacity in response to each stressor is quantified and compared, in order to determine which is the most stressful. Alternatively, a putative stressor may be tested to determine if it results in a change in the individual's physiological status which is indicative of stress.

As indicated above, methods of the invention are applicable not only to humans but also non-human mammals and additionally birds. For example, a method of the invention can be applied to farmed animals, such as cattle, pigs, sheep, lambs and poultry, e.g. chickens. Importantly, such a method may be used to assess if farmed animals are suffering from significant stress before slaughter with a view to providing meat products which might be labelled "stress-free". A method of the invention may also be applied to farmed animals with a view to providing a wide range of other products which might be labelled "stress-free" or "obtained from stress-free animals", for example, dairy products such as milk, dried milk, cheese, evaporated milk, condensed milks and ice-cream, wool from sheep and lambs tested in accordance with the invention, fine animal hair from animals such as alpacas, llamas, camels, yaks and goats tested in accordance with the invention and coarse animal hair for brush–making. A method of the invention may be applied to chickens for the purpose of obtaining eggs which might be marketed as coming from "stress-

free chickens". Methods of the invention may also be applied to farmed animals to improve animal husbandry techniques. For example, such methodology might be applied for certification of farms as "stress-free farms". Methods of the invention also find application in wildlife conservation and care as illustrated by the badger study already mentioned above and further described in Example 1 below.

Methods of the invention may be applied in assessing and improving the ergonomic efficiency of a human working environment. Thus, methods of the invention may have application in office design, furniture design, lighting, colouring and heating of a working environment and design of office equipment such as computers. Methods of the invention may additionally find application in such diverse fields as transport design, design of leisure facilities, e.g. spas and gyms, and shopping facilities to enhance well-being and reduction of stress-factors. They may also find application for certification of animal boarding houses, e.g. for cats and/or dogs, as "stress-free".

In a further aspect, the invention also provides a method of screening for a stress-relieving drug, which comprises administering a test compound to an individual; exposing the individual to a psychological stressor, and measuring their coping capacity using a method according to the invention; and comparing their coping capacity after administration of the test compound to their coping capacity in the absence of the test compound, wherein an increase in coping capacity after administration of the test compound is indicative of stress-relieving ability of said test compound.

The individual used in the above screening method may be a human or animal, for example a mouse, rat, hamster, guinea pig or other mammal. The individual is subjected to a stressor, which may be for example an unpleasant stimulus or stressful situation. In humans, the stressor may be completion of a task under time pressure, for example Raven's progressive matrices, or observing a stressful event which may be fictitious or real. In animals, the stressor may be exposure to an unfamiliar environment. For example, a rat may be placed in an unfamiliar environment such as the "elevated cross". The stressor may also be social stress, for example stress may

be induced in mice by housing them in individual cages and then introducing a stranger. Other suitable psychological stressors may be identified using a method of the invention for determining exposure to such a stressor.

Suitable test substances which can be tested in the above screening method include combinatorial libraries, defined chemical entities and compounds, peptide and peptide mimetics, oligonucleotides and natural product libraries, such as phage display libraries and antibody products. Typically, organic molecules will be screened, preferably small organic molecules which have a molecular weight of from 50 to 2500 daltons. Candidate products can be biomolecules including, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof. Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, or amidification to produce structural analogs.

A stress-relieving drug identified by a screening method as above may be synthesized and/or formulated into a pharmaceutical composition. Formulation with pharmaceutically acceptable carriers and/or excipients may be carried out using routine methods in the pharmaceutical art. Thus the manner of formulation will depend upon factors such as the nature of the substance and the condition to be treated. Any such substance may be administered in a variety of dosage forms. It may be administered, for example, orally (e.g. as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules), parenterally, subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques.

Once an individual has been diagnosed as suffering stress by a method of the invention, they may be provided with stress-relieving treatment, for example, they may be administered a known stress-relieving drug. Thus in a still further aspect, the present invention provides use of a stress-relieving drug in the manufacture of a medicament for treating an individual, e.g. a human, who has been identified as

suffering stress by a method of the invention. There is additionally provided a method of treating an individual for stress, e.g. by administration of a stress-relieving drug wherein said individual has been identified as suffering from stress by a method of the invention.

A stress-relieving drug for use in such a method may be one that reduces stress, improves or ameliorates the symptoms of stress, or a drug that treats a stress-related disorder. For example, the drug may be a known anxiolytic or antidepressant or an adjunct to such drug therapy, e.g. drugs such as tryptophan The main classes of anxiolytic drugs are benzodiazepines, for example diazepam and alprazolam; 5-$HT_{1A}$-receptor agonists, such as bupirone; and β-adrenoceptor antagonists, for example propranolol. The main classes of antidepressant drug are tricyclic antidepressants, for example imipramine and amitriptyline; selective serotonin reuptake inhibitors, for example fluoxetine, fluvoxamine, paroxetine and sertraline; monoamine oxidase inhibitors, such as phenelzine, tranylcypromine, clorgyline and moclobemide; and atypical antidepressants, for example nomifensine, maprotiline, mianserin, bupropion and trazodone.

In a still further aspect, the present invention provides a method of testing the efficacy of a proposed stress-relieving treatment which comprises exposing an individual, e.g. a human, to a psychological stressor in the presence and absence of said treatment and determining their coping capacity as described above. The treatment to be tested may be, for example, handling of a device, gadget or toy designed with the aim of bringing stress relief or exposure to a particular environment, e.g. a smell as in aromatherapy, a pattern of light and/or imagery or sound composed with a view to bringing stress relief. Efficacy of any such treatment will be indicated by an increase in challenge-induced superoxide production indicative of an increase in coping capacity

The following examples illustrate application of the invention to wild animals, in particular wild badgers, and human volunteers.

## EXAMPLES

### Example 1 – Trapping and transport of wild badgers

As indicated above, this study was carried out to test the prediction that transport of badgers causes substantial additional psychological stress compared to trapping of such animals without transport as reflected in lower coping capacity determined from residual capacity of blood neutrophils for *in vitro* activation.

Methods

***(a) Trapping and transporting badgers***

Badgers were trapped in Wytham Woods, Oxfordshire, U.K. (for details of the study site and its badger population see Macdonald & Newman (2002) J. Zool. 256, 121-138) in cage traps baited with peanuts in August and November 2001. We selected badgers because a measure of stress for this species would be immediately useful in studies of its unusual social system (Macdonald & Newman 2002) and its role in the epidemiology of bovine TB. Also, studies, conservation and control of badgers necessitate their capture and handling, so a means of evaluating alternative procedures would be helpful. We selected this population because their individual life histories have been monitored for 14 years during which our particular trapping and handling procedures have been refined to the highest welfare standards. Badger traps were set adjacent to badger setts between 1400h and 1700h. Traps were checked each morning between 0630 and 0700 and trapped badgers were transferred to individual holding cages.

Animals were then assigned to one of two experimental regimes: sampling at the site of capture, without transport (non-transported, n=8); or sampling immediately after transport (transported, n=8). Transport consisted of a short ride of less than 10 minutes on a trailer pulled by an all-terrain quad bike. While in holding cages, badgers were covered with a cloth. Badgers were anaesthetised using an intra-muscular injection of ketamine hydrochloride ("Ketaset," Fort Dodge, U.S.A.) at a

dose of 0.2 ml/kg. Processing consisted of measurements of body weight and length, and recording the sex and condition of the badger. Other measurements were also taken as part of the ongoing badger population study. Blood was removed by needle venepuncture of the jugular vein, collected into a tube containing the anticoagulant potassium EDTA (BD Vacutainer Systems, Plymouth U.K.) and taken immediately for PMA challenge. A leucocyte count ($10^9$/L) was also made for each individual using a haematology analyzer (Celltac MEK-5108K Kohden, Japan). Differential cell counts were made from blood smears fixed in alcohol and stained with May-Grunwald and Giemsa stains.

***(b) PMA challenge and measurement of Coping Capacity***

To measure the background blood chemiluminescence levels, 10 µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial E G & G Berthold Germany), to which 90 µl of $10^{-4}$M luminol (5-amino-2,3-dihydrophthalzine; Sigma A8511) was added. The tube was then shaken gently. To measure chemiluminescence produced in response to bacterial challenge, a further tube was prepared as above, but with the addition of 10µl of the microbial product Phorbol 12-Myristate 13-Acetate (Sigma P8139) at a concentration of $10^{-6}$M. For each tube luminescence was measured for 30 seconds every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold Germany) for a total of 30 minutes. When not in the chemiluminometer, tubes were incubated at 37°C.

***(c) Statistical analyses***

To compare differences in coping capacity in badgers with and without transport, multivariate analysis of variance (MANOVA) was used, with the response at each time interval as dependent variables, and transport regime as a treatment variable. This procedure was carried out on SPSS for Windows release 10.0.5. Data were log transformed prior to the analysis to ensure the data met assumptions of multivariate normality (Tabachnick & Fidell (1996), Using Multivariate Statistics, Third Edition, New York: HarperCollins College Publishers). Wilcoxon Signed Ranks Tests were used to compare an individual's Coping Capacity with its basal response.

*(d) Animal welfare considerations*

The badger population at Wytham is under long-term scientific investigation, and badgers are trapped and transported regularly throughout their lives. Examining transport stress is part of our continuing refinement of best practice. Work was carried out under English Nature licence 1991537 and Home Office licence PPL 30/1826.

Results

Basal superoxide production in and coping capacity in two groups of animals, transported ($n = 8$) and non-transported ($n = 8$), are shown in Figure 1. Transport significantly reduced coping capacity in badgers ($F_{7,8} = 4.5, p < 0.05$). The difference in coping capacity between transported and non-transported individuals was greatest at 15 minutes ($F_{7,8} = 6.6, p < 0.05$; Figure 1). However, two of the non-transported individuals were caught during a night when ambient temperatures unexpectedly fell below freezing, and these individuals did not show a typical response for this group, and in both cases their coping capacity was no greater than their basal response (Wilcoxon Signed Ranks Test, for both cases $Z < -1.0, p > 0.1$). Transport regime had no effect on basal superoxide production ($F_{7,8} = 2.5, p > 0.05$).

There were differences in the number of leucocytes between the two groups, with transported individuals having significantly lower numbers of leucocytes (Mann-Whitney $U = 11.0, p < 0.05$). In addition, leucocyte composition also differed between the groups: transported animals had a higher percentage of neutrophils (Mann-Whitney $U = 13.5, p = 0.05$), and a correspondingly lower percentage of lymphocytes (Mann-Whitney $U = 7.5, p < 0.01$). To examine leucocyte activity, we calculated coping capacity per leucocyte (Figure 2), and found the same pattern as overall coping capacity, indicating that per-leucocyte activity was greater in non-transported individuals.

These results indicate that coping capacity is a quantifiable measure of the stress associated with a specific event. The rather uniform depression of the coping

response in all transported individuals suggests that this stressor over-rode the sources of individual variation that characterised the coping responses of non-transported individuals.

**Example 2 – Observing a fictitious stressful event**

The purpose of this study was to assess the effect of watching a potentially stressful event on human volunteers.

Methods

***(a) Subjects***

Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before this study commenced, in accordance with the declaration of Helsinki. 14 subjects all moderately fit and healthy aged between 20-26 participated in the study. Exclusion criteria included suffering from psychiatric illness, respiratory or cardiovascular disease, smokers, or prescription medicine taken within the previous month.

***(b) Design***

The experiments were performed in the afternoon according to highly standardised procedures. Subjects were instructed to avoid exercise or alcohol for 48 hours before the study and be fasting for 2 hours before the study began. Both groups sat quietly for 15 minutes to obtain resting blood pressure, heart rate and resting blood samples. Blood samples were then taken from all subjects 15 minutes before being exposed to either no stress (control conditions) or psychological stress in the in the form of exposure to the horror film. This time point was designated -15 minutes. Subjects had no prior knowledge as to which group they would be assigned to.

8 subjects were exposed to psychological stress in the form of an 83-minute horror film that none of the subjects had previously seen (The Texas Chainsaw Massacre, 1974, directed and produced by Tobe Hooper, Everett Collection, Inc). The remaining 6 subjects acted as a control group. They were instructed to sit quietly for

the same length of time (83 minutes), under similar lighting conditions and were given emotionally non-stimulating material to read if they so wished. This consisted of text books, articles from journals and health information leaflets. A repeated measures design was implemented with two groups; the experimental group (watching the horror film) and a control group. Blood samples, heart rate and blood pressure measurements were taken 15 minutes before, and just after (85 minutes) being exposed to either the 'stressful event' or to control conditions.

***(c) Heart rate & blood pressure measurements***

A heart rate transceiver (Polar, Heart rate monitor) was attached directly to the chest and heart rate was monitored. Participants were seated, asked to make themselves comfortable, close their eyes and breathe orthonasally. This procedure was carried out for a period of fifteen minutes, in order to minimise possible stress levels experienced prior to, or upon arrival, at the laboratory. At the end of this period baseline heart rate was recorded and the first blood sample taken (see sample protocol below). Participants were then instructed to either read quietly or in a separate room to watch the horror film. Upon completion of the task heart rate was recorded again, and further blood samples taken. Systemic blood pressure (BP) was measured using an aneroid sphygmomanometer (Accoson, (Surgical) Ltd, London) and stethoscope (Harvard Ltd, Edenbridge, UK). Finger stick samples of blood 20 µl were obtained at the specified time points.

***(d) PMA challenge and measurement of Coping Capacity***

To measure the background blood chemiluminescence levels, 20µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial E G & G Berthold Germany), to which 90 µl of $10^{-4}$M luminol (5-amino-2,3-dihydrophthalzine; Sigma A8511) was added. The tube was then shaken gently. To measure chemiluminescence produced in response to bacterial challenge, 20 µl of the microbial product Phorbol 12-Myristate 13-Acetate (Sigma P8139) at a concentration of $10^{-3}$M was added. For each tube luminescence was measured for 30 seconds every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold Germany) for a total of 30 minutes. The maximum reponse to PMA When

not in the chemiluminometer, tubes were incubated at 37°C. The maximum response to PMA was noted.

*(e) Data analyses.*

Data are expressed as means ± standard deviation from mean. A 2-tailed unpaired t-test was subsequently used to compare the 2 groups at baseline and 85 minutes.

Results

Table 1 shows the differences in the response to PMA between the horror film group (n=8) and the control group (n=6). A highly statistically significant ($p < 0.05$) increase was found at 85 minutes in the horror film group when compared to the control group. The average heart rate change of +14 for the horror film group represents a 20.6% increase. Their systolic and diastolic blood pressure rose by 10 % and 16 % respectively. The control group showed no such changes.

| Max response to PMA (RLU) | -15 minutes Baseline | 85 minutes |
|---|---|---|
| Control | 320 (40) | 340 (35) |
| Horror group | 325 (27) | 120 (60)* |

Table 1: Summary of max response to PMA. SD in brackets. *P<0.05 from control. RLU = Relative Light Units

Watching a horror movie elicits a psycho physiological arousal which is comparable to Canon's fear flight fight defence reaction, the so called 'stress response' which involves stimulation of the hypothalamus, a change in peripheral resistance and an increase in the release of stress hormones including catecholamines and cortisol. This study also shows that stress affected the responsiveness of a peripheral sample of blood. This can therefore be used as an indictor of stress.

An altered responsiveness to PMA indicates *in vivo* changes to the state of leukocyte activation. In a state of stress, it is possible that the leukocytes actually release the contents of their granules (and are thus are unable to respond to PMA) having already released the contents of their granules. Such leukocytes would be unable to respond to opportunistic infections thus rendering the host more susceptible to disease, as well as potential tissue damage from a host of proteolytic enzymes and oxygen free radicals.

**Example 3 – Stressor task**

Method

***(a) Subjects***

Local ethical committee approval from Coventry University Ethics Committee and informed consent was obtained before this study commenced, in accordance with the declaration of Helsinki. 12 undergraduate students (aged 18-23 years) were assigned to either an experimental group (given test) or asked to sit quietly for an equivalent time period. Exclusion criteria included suffering from psychiatric illness, respiratory or cardiovascular disease, smokers, or prescription medicine taken within the previous month.

***(b) Stressor task***

The test was a written visual spatial task test comprising 36 questions, on which a 15 minute constraint was imposed (Ravens J. C. Advanced Progressive Matrices, set II, Oxford Psychologists Press, Oxford, 1994). A small finger stick blood sample (Boehringer Mannheim, Soft Clix) was taken before and immediately after task completion. 20 µl of blood was collected in a pipette.

***(c) PMA challenge and measurement of Coping Capacity***

To measure the background blood chemiluminescence levels, 20 µl of whole blood was transferred into a silicon anti-reflective tube (Lumivial E G & G Berthold

Germany), to which 90µl of $10^{-4}$M luminol (5-amino-2,3-dihydrophthalzine; Sigma A8511) was added. The tube was then shaken gently. To measure chemiluminescence produced in response to bacterial challenge, 20µl of the microbial product Phorbol 12-Myristate 13-Acetate (Sigma P8139) at a concentration of $10^{-3}$M was added. For each tube luminescence was measured for 30 seconds every five minutes in a portable chemiluminometer (Junior LB 9509 E G & G Berthold Germany) for a total of 30 minutes. When not in the chemiluminometer, tubes were incubated at 37°C. The maximum response to PMA was noted.

***(d) Data analyses***

Data are expressed as means ± standard deviation from mean. A 2-tailed unpaired t-test was subsequently used to compare the 2 groups at baseline and 15 minutes.

Results

Table 2 shows the effect of completing a stressor task on leukocyte responsiveness to PMA. The experimental group had significantly reduced responses to PMA ($p<0.05$, unpaired t test) compared to controls. Therefore completion of a stressor task diminished responsiveness to PMA.

| Max response to PMA (RLU) | Baseline | 15 minutes |
|---|---|---|
| Control | 420 (40) | 380 (25) |
| Experimental Group | 380 (30) | 160 (70)* |

Table 2 Summary of max response to PMA. Mean +/- Standard deviation (SD) in brackets . *P<0.05 from control. RLU = Relative Light Units

## CLAIMS

1. A method for determining whether an individual, which is a mammal or bird, is experiencing changed physiological status arising from exposure to a psychological stressor, the method comprising:

(a) contacting a test sample comprising neutrophils obtained from said individual with an inducer capable of stimulating superoxide production in neutrophils, under conditions suitable for such stimulation;

(b) determining the increase in superoxide production above basal in said test sample after a time period when neutrophils of the same species in a control sample, which are free or substantially free of stress-induced activation or at least derived from one or more individuals exposed to the same regime minus a factor to be tested as a psychological stressor, will exhibit superoxide production under the same *in vitro* conditions; and

(c) comparing the increase in superoxide production above basal observed in said sample with the increase in superoxide production above basal observed in a control sample as defined in (b) above under the same conditions;

wherein lower superoxide production in said test sample is indicative of the effect of a psychological stressor on the individual's physiological status.

2. A method according to claim 1 for determining the coping capacity of an individual for exposure to a psychological stressor, wherein prior to step (a) said individual is exposed to said psychological stressor for a time period whereby neutrophils in an individual of the same species who is susceptible to stress induced by said stressor will exhibit increased superoxide production and wherein the degree of further *in vitro* induced superoxide production in said test sample above basal determined in step (c) is a measure of coping capacity.

3. A method according to claim 1 or claim 2 wherein said sample comprises isolated leucocytes

4. A method according to claim 1 or claim 2 wherein said sample is a whole blood sample.

5. A method according to any one of claims 1 to 4, wherein the individual is human.

6. A method according to any one of claims 1 to 4 wherein the individual is a bird, such as a chicken.

7. A method according to any one of claims 1 to 4 wherein the individual is a farmed animal, such as a cow, pig, sheep, lamb or poultry.

8. A method according to any of the preceding claims, wherein the inducer capable of stimulating superoxide production in neutrophils is phorbol myristate acetate (PMA), N-Formyl-Met-Leu-Phe (FLMP chemotactic peptide), zymosan, lipopolysaccharide or adrenaline.

9. A method according to any of the preceding claims, wherein superoxide production is detected using luminol or isoluminol as an amplifier and the resulting chemiluminescence is measured.

10. A method according to claim 1 or claim 2, wherein the inducer capable of stimulating superoxide production in neutrophils is phorbol myristate acetate (PMA), superoxide production is detected using luminol as an amplifier and the resulting chemiluminescence is measured.

11. A method of screening for a stress-relieving drug, the method comprising:
    (a) administering a test compound to an individual;
    (b) exposing said individual to a psychological stressor and measuring their coping capacity using a method according to any of claims 2 to 10; and

# ABSTRACT

## QUANTIFYING EXPOSURE TO STRESS

The present invention provides an *in vitro* method for quantifying exposure to psychological stress which relies on measuring the retained ability of neutrophils, preferably neutrophils in a whole blood sample, to exhibit challenge-induced superoxide anion production. Using such methodology, coping capacity of individuals for particular psychological stressors may be assessed.

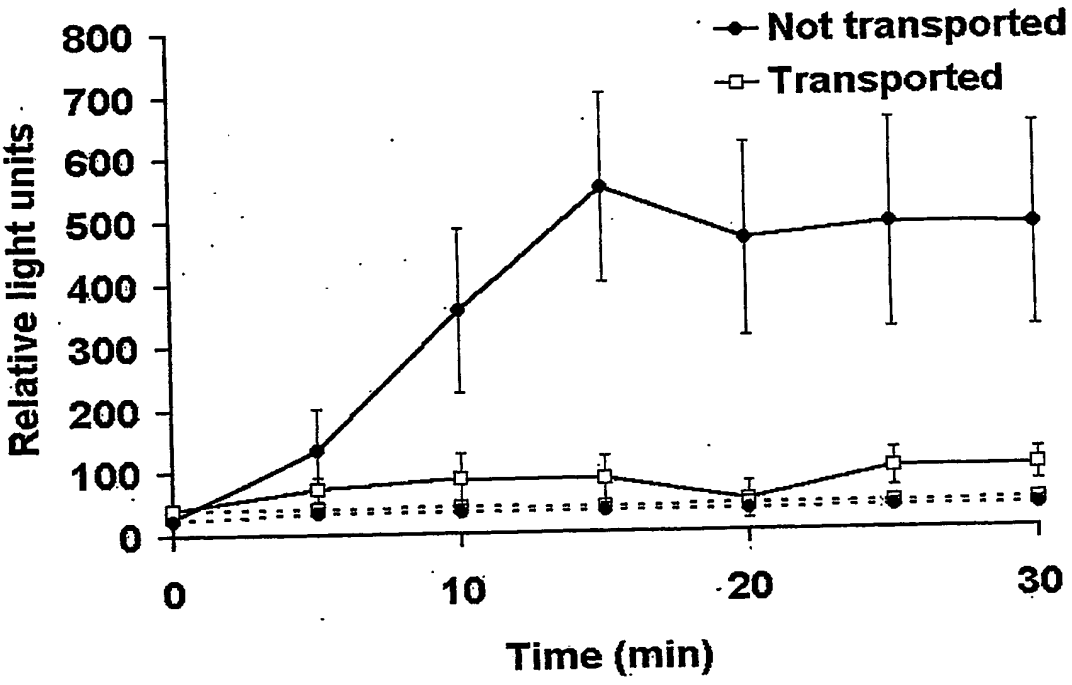

Not transported
Transported
Relative light units
800
700
600
500
400
300
200
100
0
0
10
20
30
Time (min)

Figure 1

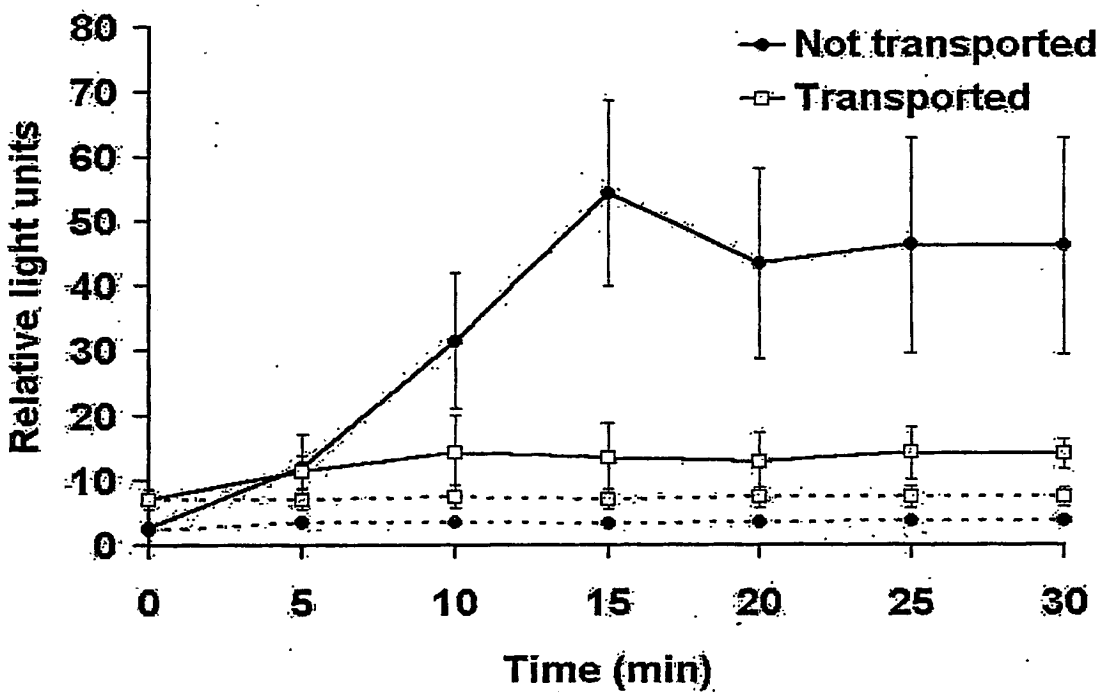

Not transported
Transported
Relative light units
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (min)

Figure 2